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Differential Gene Expression for Investigation of *Escherichia coli* Biofilm Inhibition by Plant Extract Ursolic Acid

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After 13,000 samples of compounds purified from plants were screened, a new biofilm inhibitor, ursolic acid, has been discovered and identified. Using both 96-well microtiter plates and a continuous flow chamber with COMSTAT analysis, 10 μg of ursolic acid/ml inhibited *Escherichia coli* biofilm formation 6- to 20-fold when added upon inoculation and when added to a 24-h biofilm; however, ursolic acid was not toxic to *E. coli*, *Pseudomonas aeruginosa*, *Vibrio harveyi*, and hepatocytes. Similarly, 10 μg of ursolic acid/ml inhibited biofilm formation by >87% for *P. aeruginosa* in both complex and minimal medium and by 57% for *V. harveyi* in minimal medium. To investigate the mechanism of this nontoxic inhibition on a global genetic basis, DNA microarrays were used to study the gene expression profiles of *E. coli* K-12 grown with or without ursolic acid. Ursolic acid at 10 and 30 μg/ml induced significantly ($P < 0.05$) 32 and 61 genes, respectively, and 19 genes were consistently induced. The consistently induced genes have functions for chemotaxis and mobility (*cheA*, *tap*, *tar*, and *motAB*), heat shock response (*hslSTV* and *mopAB*), and unknown functions (such as *b1566* and *yrfHI*). There were 31 and 17 genes repressed by 10 and 30 μg of ursolic acid/ml, respectively, and 12 genes were consistently repressed that have functions in cysteine synthesis (*cysK*) and sulfur metabolism (*cysD*), as well as unknown functions (such as *hdeAB* and *yhaDFG*). Ursolic acid inhibited biofilms without interfering with quorum sensing, as shown with the *V. harveyi* AI-1 and AI-2 reporter systems. As predicted by the differential gene expression, deleting *motAB* counteracts ursolic acid inhibition (the paralyzed cells no longer become too motile). Based on the differential gene expression, it was also discovered that sulfur metabolism (through *cysB*) affects biofilm formation (in the absence of ursolic acid).

Bacterial biofilms are sessile communities with high cell density that are ubiquitous in natural, medical, and engineering environments (14, 42). Biofilms formed by pathogenic *Escherichia coli* strains can pose serious problems to human health such as prostatitis, biliary tract infections, and urinary catheter cystitis (8). Deleterious biofilms are also problematic in industry since they cause fouling and corrosion in systems such as heat exchangers, oil pipelines, and water systems (14). Recently, there has been a tremendous increase in biofilm research, most of it with the ultimate aims of biofilm prevention, control, or eradication (32).

Biofilm cells survive antibiotics more readily than planktonic ones and are often responsible for reoccurring symptoms and medical treatment failure (39, 42). Because bacterial cells in a biofilm are embedded in a matrix of polysaccharide, they encounter oxygen limitation and low metabolic activity, which protect them from antibiotics (66). In addition, it was found that 40% of the cell wall proteins in biofilm cells are different from those of the planktonic cells; therefore, some antibiotics may lose their targets (42). Since many cells in a mature biofilm live extended times without division, they are highly resistant to antibiotics which are primarily effective on dividing cells (42). For these reasons, biofilms are highly resistant to antibiotics (39); hence, novel antagonists with potential to remove mature biofilms are needed. By concentrating on novel antagonists that do not inhibit growth, we seek to avoid selection pressure for resistance (21).

There are few known natural compounds that inhibit biofilm formation while not affecting cell growth, but the quorum-sensing antagonist (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone (furanone) from the marine alga *Delisea pulchra* (12) inhibits biofilm formation in *E. coli* without inhibiting its growth (51). Bacteria use quorum sensing to regulate some forms of gene expression by sensing their population density via the small signaling compounds called autoinducers (AIs) that are excreted into the environment (2). As the AI concentration increases with cell density, the binding of AIs to the cellular receptors triggers genes for different phenotypes including biofilm formation (9), production of virulence factors (4), siderophore synthesis (62), bioluminescence (7), protein production (10), and plasmid conjugation (35). Different species use different quorum-sensing signals; however, AIs are mainly divided into two groups: acylated homoserine lactones (AI-1, regulated by LuxI/LuxR systems) for gram-negative bacteria and peptides for gram-positive bacteria (2). A common signal called AI-2 (produced by LuxS) has been discovered as a species nonspecific signal used by both gram-negative and gram-positive bacteria (64). Sperandio et al. used DNA mi-

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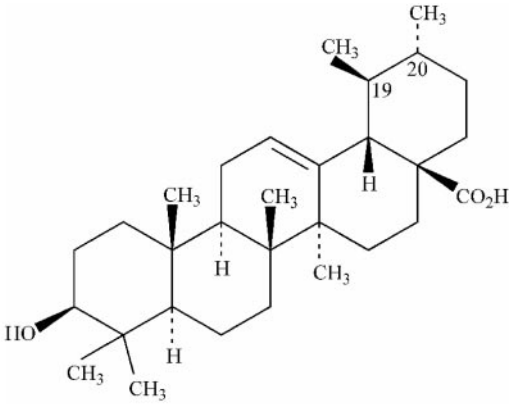

FIG. 1. Structure of ursolic acid. Carbons 19 and 20 are shown.

croarrays to study gene expression of wild-type *E. coli* O157:H7 and its *luxS* mutant and found that AI-2 is a global regulatory signal which regulates more than 400 genes, including those for chemotaxis, flagellar synthesis, motility, and virulence factors (58). Using DNA microarrays, furanone has been shown by us to repress 44 of the 67 *E. coli* genes that are positively controlled by AI-2 (49), and Hentzer et al. (22) have shown that brominated furanone also inhibits 80% of the AI-1 quorum-sensing-controlled genes in *Pseudomonas aeruginosa* PAO1.

To search for additional natural compounds that inhibit biofilm formation without affecting growth, a library of compounds from 176 plant families was created (13). From this library, ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) from *Diospyros dendo*, the tree used for ebony (e.g., black piano keys) from Gabon, Africa, was identified (Fig. 1). Ursolic acid is a relatively nontoxic active ingredient of many medicinal plants such as *Sambucus chinesis*, *Glechoma hederacea*, *Eribotrya japonica*, and *Pyrola rotundifolia* and has a broad range of pharmacology effects, including protection against liver injury, antitumor activity, inhibition of mutagenesis in bacteria, anti-inflammation, and antiulcer activity (36). Here, by using 96-well plates and a flow chamber, ursolic acid is shown to inhibit biofilm formation of the reference microorganism *E. coli* without affecting its growth rate. To investigate the genetic basis of this biofilm inhibition, DNA microarrays, which have been used to monitor global gene expression profiles in response to different stimuli (57) including heat shock and other stresses (20, 70, 73), quorum sensing (11, 58), anaerobic metabolism (72), sporulation (15), and biofilm formation (46, 47, 55, 60, 69), were used to study the differential gene expression of *E. coli* K-12 with or without ursolic acid. This is the first report of using ursolic acid to inhibit biofilms and of the role of sulfur metabolism in biofilm formation.

MATERIALS AND METHODS

Bacterial strains, growth media, and toxicity testing. The strains and plasmids used in the present study are listed in Table 1 (including two *E. coli* K-12 MG1655 strains for use with their respective mutants). LB medium (54) was used for routine culturing of the *E. coli* strains. LB medium and LB medium supplemented with 0.2% glucose, 1% sodium citrate, or 2% sodium pyruvate was also used to study biofilm formation in the 96-well plate assay. M9 medium (53) supplemented with 0.4% glucose and 0.4% Casamino Acids was used to establish the *E. coli* biofilm in the flow chamber and to study biofilm formation by *Vibrio harveyi* in the microtiter plates. Autoinducer bioassay (AB) medium (18) was used to grow *V. harveyi*, and LM medium (3) was used to determine its CFU. Plasmids were electroporated at 15 kV/cm, 200 Ω, and 25 μF by using a Gene Pulser (Bio-Rad, Richmond, CA).

The specific growth rates of *E. coli* K-12 ATCC 25404 with or without conjugation plasmid R1*drd19* in the presence of 10 or 30 μg of ursolic acid/ml in LB medium were determined by using the optical density at 600 nm (OD_{600}) and calculated by using the linear portion of the logarithm of OD_{600} versus time (OD_{600} from 0.05 to 0.4). Similarly, the effects of the *cysB* and *motAB* mutations on growth rates were determined. Hepatocyte toxicity testing with 13.7 μg of ursolic acid/ml (30 μM) was performed by Cerep (Redmond, WA) according to the method of Nociari et al. (40) using a sensitive fluorometric assay based on AlamarBlue and the HepG2 cell line.

Plant collection and purification of ursolic acid. To discover novel biofilm inhibitors, plant extracts were separated by high-pressure liquid chromatography (HPLC) and screened with *P. aeruginosa* PAO1 biofilm assays by using microtiter plates and staining with crystal violet. Fractions that inhibited biofilm formation were purified, and the component compounds were tested individually. The plants were collected from Gabon, Africa, and the Missouri Botanical Garden at St. Louis, Mo. A total of 13,000 fractions (6,699 from Missouri and 5,941 from Gabon) were screened, representing 83 families and 167 genera. The plant collection included whole plants and separated parts such as fruits, roots, leafs, and stems (13). From these fractions ursolic acid was identified. Ursolic acid (Fig. 1) was extracted by using preparative HPLC from a leaf/flower extract from *D. dendo* as described previously (13). This process differs from traditional biologically guided fractionation because there was only one purification step. The separation was optimized to achieve baseline resolution of ursolic acid and the other compounds in the sample mixture. The HPLC method used to obtain ursolic acid consisted of a linear gradient of acetonitrile from 75 to 95% in 28 min on a semipreparative C_{18} column. Ursolic acid was eluted at 21.2 min and stored as a powder at −20°C. Within 48 h prior to the experiment, ursolic acid was dissolved in 95% ethanol at 2.5 mg/ml and stored at 4°C.

Total RNA isolation for DNA microarrays. To identify the genes controlled by ursolic acid, *E. coli* K-12 ATCC 25404 was grown in LB medium overnight, diluted 1:100 in fresh LB supplemented with 0, 10, or 30 μg of ursolic acid/ml. The same amount of ethanol was supplemented to eliminate solvent effects. The cultures were grown to an OD_{600} of 0.9. The cells were centrifuged in a microcentrifuge for 15 s at 20,000 × *g* in Mini-Bead Beater tubes (Biospec, Bartlesville, Oklahoma) that were cooled to −80°C before sampling. The cell pellets were flash frozen in a dry ice-ethanol bath and stored at −80°C until RNA isolation.

To lyse the cells, 1.0 ml of RLT buffer (Qiagen, Inc., Valencia, CA) and 0.2 ml of 0.1 mm zirconia-silica beads (Biospec) were added to the frozen bead beater tubes containing the cell pellets. The tubes were closed tightly and beat for 30 s at the maximum speed in a Mini-Bead Beater (catalog no. 3110BX; Biospec). The total RNA was isolated by following the protocol of the RNeasy Minikit (Qiagen), including an on-column DNase digestion with RNase-free DNase I (Qiagen). OD_{260} was used to quantify the RNA yield. 23S/16S rRNA values (measured by using a 2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA) and OD_{260} and OD_{280} values were used to check the purity and integrity of RNA (RNeasy Mini handbook; Qiagen).

DNA microarrays. The *E. coli* DNA microarrays were prepared as described previously (68). Each gene probe was synthesized by PCR and has the size of the full open reading frame (ORF; 200 to 2,000 nucleotides). The double-strand PCR products were denatured in 50% dimethyl sulfoxide and spotted onto aminosilane slides (Full Moon Biosystems, Sunnyvale, CA) as probes to hybridize with the mRNA-derived cDNA samples. It has been shown that each array can detect 4,228 of the 4290 *E. coli* ORFs (68). Each gene has two spots per slide.

The detailed microarray protocol has been described in our recent publication (50). Briefly, the total RNA from the *E. coli* K-12 ATCC 25404 samples grown with or without ursolic acid was first converted into labeled cDNA. The cDNA samples (6 μg of each) were then each labeled with both Cy3 and Cy5 dyes to remove artifacts related to different labeling efficiencies; hence, each experiment needed at least two slides. The Cy3-labeled sample without ursolic acid and the Cy5-labeled ursolic acid sample (with 10 or 30 μg of ursolic acid/ml) were hybridized on the first slide. Similarly, the Cy5-labeled sample without ursolic acid and the Cy3-labeled ursolic acid sample were hybridized on the second slide. Since each gene has two spots on a slide, the two hybridizations generated eight datum points for each gene (four points for the sample without ursolic acid and four points for the ursolic acid sample). The microarray experiments with dye-swapping were repeated for both concentrations of ursolic acid.

The labeled cDNA samples were hybridized to the DNA microarrays and scanned, and the images were analyzed as described previously (50). Genes were identified as differentially expressed if the expression ratio was >1.4 and the *P* value (*t* test) was <0.05. *P* values were calculated on log-transformed, normalized

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype[a]	Source or reference
Strains		
V. harveyi BB120	Wild type	63
P. aeruginosa PAO1	Wild type	24
E. coli K-12	Wild type	ATCC 25404
E. coli K-12 MG1655	F^- lambda$^-$ *ilvG*$^-$ *rfb-50 rph-1*	6, 43
E. coli K-12 MG1655 *motAB*	Δ*motAB uvrC*::Tn*10*	43
E. coli K-12 MG1655 *cysB*	Δ*cysB*::Tn*5*Kan-2	29
E. coli JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	71
E. coli MC4100	*araD139* Δ(*argF-lac*) *U169 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301*	34
E. coli MC4100 *cysB*	*araD139* Δ(*argF-lac*) *U169 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301 cysB*	34
E. coli C600	*thi-1 thr-1 leuB6 lacY1 tonA21 supE44 rfbD1*	34
E. coli C600 *cysB*	*thi-1 thr-1 leuB6 lacY1 tonA21 supE44 rfbD1 cysB*	34
E. coli EJ500	Prototrophic, constitutive flagellar synthesis, selected from W3110 for motility (26)	27
E. coli JCB495	MC1000 *recD*	65
Plasmids		
R1*drd19*	*finO*; Amp^r Km^r Cm^r Sm^r; IncFII (conjugation plasmid to promote biofilm formation)	45
pCM18	Em^r; pTRKL2-P_{CP25}-RBSII-*gfp*mut3*-T_0-T_1 (GFP plasmid for visualizing biofilm)	19

[a] Amp^r, Km^r, Cm^r, Sm^r, and Em^r are ampicillin, kanamycin, chloramphenicol, streptomycin, and erythromycin resistance, respectively.

intensities. Including the *P* value criterion ensures the reliability of the induced or repressed gene list. Normalization was relative to the median total fluorescent intensity per slide per channel. The gene functions were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/).

Autoinducer activity assay. Bacterial supernatants were assayed by using the method of Surette and Bassler (63), as we described previously (51). Reporter strains *V. harveyi* BB170 or BB886 were grown in AB medium overnight and diluted 1:5,000 into the fresh AB medium supplemented with 0, 1, 5, or 10 μg of ursolic acid/ml. The time course of bioluminescence was measured with a 20/20 luminometer (Turner Design, Sunnyvale, CA) and reported as relative light units (RLU). The cell density of the *V. harveyi* reporter strains was measured by spreading the cells on LM plates and counting CFU after 24 h.

Ninety-six-well plate biofilm assay. This assay was adapted from those reported previously (33, 43). *E. coli* was grown in polystyrene 96-well plates at 37°C for 2 days without shaking in LB medium (or in LB medium supplemented with 0.2% glucose or other carbon sources according to the requirements of the individual strains) supplemented with 5, 10, or 30 μg of ursolic acid/ml as indicated. The appropriate amount of 95% ethanol was added to all samples to eliminate solvent effects (each well contained 1.2% [vol/vol] ethanol). Before the biofilm mass in each well was measured, the OD reading at 620 nm was taken to quantify the growth of the cells with or without ursolic acid. To quantify the total biofilm mass, the suspension cultures were decanted, the plates were washed three times with water, and the biofilms were stained with 0.1% crystal violet (Fisher, Hanover Park, IL) for 20 min. The extra dye was removed by three washes with water. All of the dye associated with the attached biofilm (air-liquid interface biofilm as well as bottom liquid-solid biofilm) was dissolved with 300 μl of 95% ethanol, and an OD reading at 540 nm was used to quantify the total biofilm mass. Each datum point was averaged from four replicate wells and the standard deviations were calculated (Fig. 2; see also Fig. 4). To study the overall effect of ursolic acid, it was added upon inoculating *E. coli*, and a time course of biofilm mass was measured. To study whether ursolic acid can remove mature biofilms, it was also added 24 h after inoculation. One plate was processed every 8 to 24 h so that a total of four plates were prepared for each condition to give a time course, and the experiments were conducted twice (eight plates total); these experimental conditions apply for strains *E. coli* K-12 ATCC 25404 [R1*drd19*], K-12 MG1655 with or without R1*drd19*, K-12 MG1655 *motAB* with or without R1*drd19*, K-12 MG1655 *cysB* [R1*drd19*], JM109, EJ500, MC4100 [R1*drd19*], MC4100 *cysB* [R1*drd19*], C600 [R1*drd19*], and C600 *cysB* [R1*drd19*]. For *P. aeruginosa* PAO1, 1% sodium citrate was added into LB medium to promote biofilm formation since *P. aeruginosa* PAO1 made a very poor biofilm in LB medium (biofilm increased fourfold upon addition of citrate); similarly, 1% sodium citrate was used with M9 minimal medium to promote biofilm formation (only carbon and energy source). For *V. harveyi* BB120, the medium was M9 minimal medium supplemented with 0.4% glucose, 0.4% Casamino Acids, and 10 μg of thiamine/ml since *V. harveyi* BB120 made a very poor biofilm in LB medium.

Flow chamber biofilm experiments and image analysis. M9–0.4% glucose–0.4% Casamino Acids medium supplemented with 30 μg of chloramphenicol/ml and 150 μg of erythromycin/ml to maintain R1*drd19* and pCM18, respectively, was used to grow biofilm at 37°C in a continuous flow chamber (BST model FC81; Biosurface Technologies, corp., Bozeman, MT) with chamber dimensions of 2.54 by 12.7 by 50.8 mm. The flow cell contains a standard glass microscope slide on one side and a glass coverslip on the other side. An overnight culture of *E. coli* K-12 ATCC 25404 [R1*drd19*, pCM18] in LB medium supplemented with 30 μg of chloramphenicol/ml and 150 μg of erythromycin/ml was centrifuged and resuspended in the M9 medium. This diluted culture (OD_{600} = 0.05) was allowed to flow into the flow chamber for 2 h at a flow rate of 10 ml/h (in laminar flow) before the fresh M9 medium (supplemented with chloramphenicol and erythromycin) flow was started at the same flow rate. Ursolic acid was added at both inoculation and in the fresh medium at 10 μg/ml to investigate its effect on biofilm formation. The biofilm development in flow chamber was monitored by using a Leica TCS SP2 scanning confocal laser microscope (Leica Microsystems, Heidelberg, Germany) with a 40× objective lens.

Color confocal flow chamber images were converted to gray scale by using Image Converter (Neomesh Microsystems, Wainuiomata, Wellington, New Zealand). Biomass, substratum coverage, surface roughness, and mean thickness were determined by using COMSTAT image-processing software (23) written as a script in Matlab 5.1 (The MathWorks) equipped with the Image Processing Toolbox. Thresholding was fixed for all images stacks. At each time point, 3 to 15 different positions were randomly chosen for microscope analysis and 30 to 100 images were processed for each position; in all, 26 positions with 1,597 images were obtained for the biofilm without ursolic acid, and 58 positions with 2,740 images were obtained for the biofilm with ursolic acid. Values are means of data from the different positions at the same time point, and standard deviations were calculated based on these mean values for each position. Simulated three-dimensional images were obtained by using IMARIS (BITplane, Zurich, Switzerland). Fifty pictures were processed for each three-dimensional image.

Chemotaxis. To investigate whether ursolic acid is a chemoattractant or a chemorepellent for *E. coli*, a chemotaxis experiment was performed based on the protocol of Adler (1). *E. coli* K-12 ATCC 25404 was grown overnight in chemo-

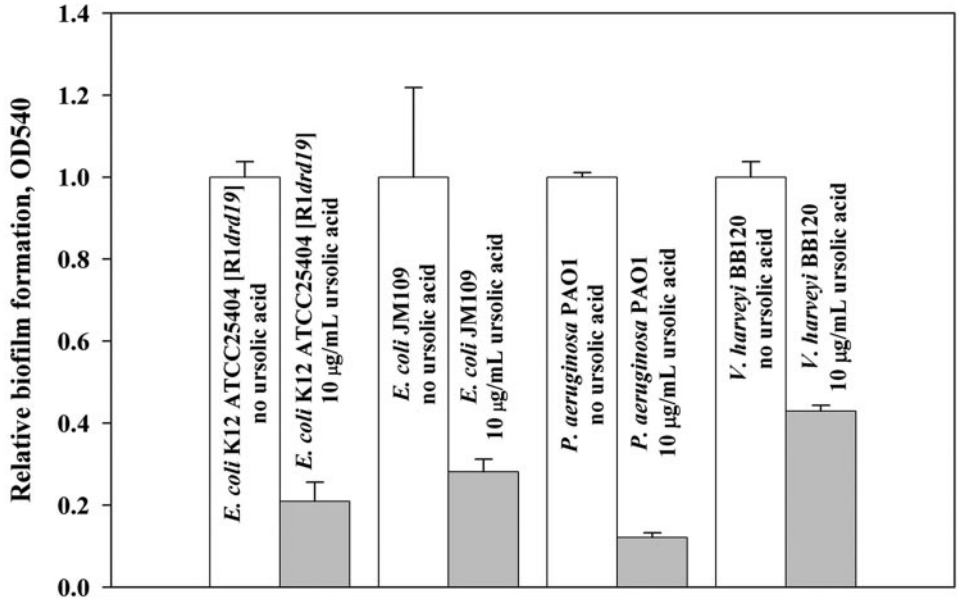

FIG. 2. Inhibition of biofilm formation by *E. coli* K-12 ATCC 25404 [R1*drd19*], *E. coli* JM109, *P. aeruginosa* PAO1, and *V. harveyi* BB120 by the addition of 10 μg of ursolic acid/ml. For *E. coli* K-12 ATCC 25404 [R1*drd19*], data were collected 16 h after the addition of ursolic acid in LB medium; for *E. coli* JM109, data were collected 24 h after the addition of ursolic acid in LB medium supplemented with 0.2% glucose**;** for *P. aeruginosa* PAO1, data were collected 18 h after the addition of ursolic acid in LB medium plus 1% sodium citrate; and for *V. harveyi* BB120, data were collected 18 h after the addition of ursolic acid in M9 medium. All biofilm mass readings at OD_{540} were normalized based on the reading of wild type without ursolic acid, which was normalized to 1. One standard deviation shown.

taxis growth medium containing 5% glycerol as a carbon source with shaking at 30°C (1). The overnight culture was diluted in the same fresh medium to an OD_{600} of 0.05 and incubated at 30°C with shaking. The culture was harvested at an OD_{600} of 0.38 and washed three times with chemotaxis buffer. To prepare the agar plug, 100 mg of Seakem Gold agarose (FMC BioProducts, Rockland, ME) was added to 5 ml of chemotaxis buffer and mixed. Then, 0.5 ml of the agarose solution and ursolic acid (30 or 100 μg/ml) were added to a microcentrifuge tube, followed by incubation at 70°C (this is the agarose plug mixture). Next, 10 μl of the above agarose plug mixture was placed between two coverslips on a microscope slide (warmed at 30°C first) and immediately covered with a third coverslip to make an agarose plug. After 4 min, 100 μl of cells was added to the agar plug, followed by incubation at 30°C for 0.5 to 1 h. The plug was examined under the microscope (Zeiss Axioskop; Zeiss, Oberkochen, Germany).

RESULTS

Ursolic acid does not inhibit the growth of *E. coli*, *P. aeruginosa*, *V. harveyi*, or hepatocytes. Ursolic acid was tested for its impact on the growth of *E. coli* K-12 ATCC 25404 in LB medium. The specific growth rate was $1.08 \pm 0.02\ h^{-1}$ without ursolic acid, $1.11 \pm 0.07\ h^{-1}$ with ursolic acid at 10 μg/ml, and $1.04 \pm 0.13\ h^{-1}$ with ursolic acid at 30 μg/ml; hence, ursolic acid is not toxic to *E. coli*. Ursolic acid also did not affect the growth rate of *E. coli* K-12 ATCC 25404 [R1*drd19*] (e.g., the specific growth rate was $1.29 \pm 0.12\ h^{-1}$ without ursolic acid, $1.36 \pm 0.07\ h^{-1}$ with ursolic acid at 10 μg/ml, and $1.16 \pm 0.08\ h^{-1}$ with ursolic acid at 30 μg/ml). Further, ursolic acid also did not affect the growth yields of JM109 in both LB medium and LB medium supplemented with 0.2% glucose in 96-well plates (5 and 10 μg/ml) and did not affect the growth yield of both wild-type *V. harveyi* (5 and 10 μg/ml in AB minimal medium and in 96-well plates) and wild-type *P. aeruginosa* PAO1 (5 and 10 μg/ml in LB supplemented with 1% sodium citrate in 96-well plates). Ursolic acid at 13.7 μg/ml was also not toxic to hepatocytes since it inhibited *HepG2* cell viability by only 1%. These results are similar to those found with oleanolic acid, which is also a triterpenoid that differs from ursolic acid by a methyl group at positions 19 and 20 (Fig. 1) (36). Hence, ursolic acid was not toxic at least at 10 μg/ml for all of the tested organisms. However, it significantly inhibited bacterial biofilm formation at 10 μg/ml.

Ursolic acid inhibited *E. coli* biofilm formation in 96-well plates. To study the effects of ursolic acid on *E. coli* biofilm formation, plasmid R1*drd19* was electroporated into *E. coli* K-12 ATCC 25404 to promote biofilm formation since this strain makes a better biofilm in the presence of conjugation pili (16, 45). Biofilm formation was inhibited clearly in LB medium when ursolic acid was added to a 24-h biofilm (Fig. 2). The biofilm without ursolic acid increased with time until 40 h after inoculation and then became stable. For the wells that contained 10 μg of ursolic acid/ml, however, the biofilm decreased by 79% 16 h after addition of the ursolic acid (Fig. 2). Also, 30 μg of ursolic acid/ml decreased biofilm formation by 40% in M9 medium supplemented with glucose. Similarly, biofilm formation was inhibited by 87% for *P. aeruginosa* PAO1 in complex medium and 95% in minimal medium and by 57% for *V. harveyi* in minimal medium (Fig. 2).

Other *E. coli* strains and media were also tested to investigate the effects of ursolic acid on biofilm formation, including its effect on a relatively good biofilm-forming strain, JM109. Ursolic acid was found to inhibit the biofilm formation of *E. coli* K-12 ATCC 25404 [R1*drd19*] in LB supplemented with 0.2% glucose (51% reduction observed 39 h after the addition of 10 μg/ml), of *E. coli* JM109 in LB (62% reduction observed 24 h after the addition of 10 μg/ml) and LB supplemented with

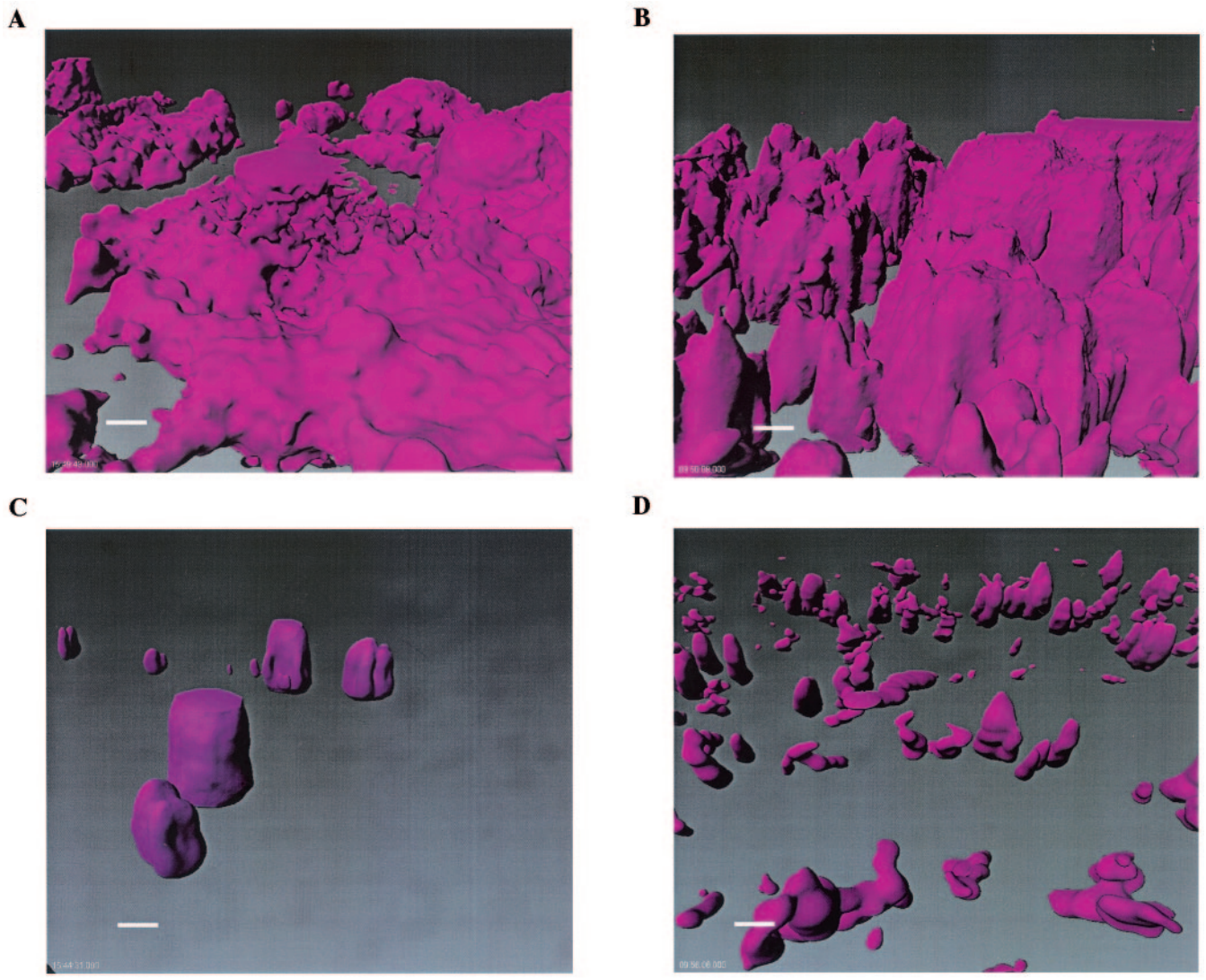

FIG. 3. Inhibition of *E. coli* K-12 ATCC 25404 [R1*drd19*, pCM18] biofilm development in flow cells upon the addition of 10 μg of ursolic acid/ml at inoculation to M9 medium (supplemented with 0.4% glucose and 0.4% Casamino Acids). (A) No ursolic acid, 22 h; (B) no ursolic acid, 65 h; (C) 10 μg ursolic acid/ml, 22 h; (D) 10 μg ursolic acid/ml, 65 h. Scale bar, 10 μm.

0.2% glucose (72% reduction at 24 h after the addition of 10 μg/ml, Fig. 2), and of *E. coli* EJ500 in LB and LB supplemented with 0.2% glucose (92% reduction at 23 h after the addition of 10 μg/ml to both media), as well as to inhibit the biofilm of *E. coli* JCB495 in LB supplemented with 2% sodium pyruvate to promote biofilm formation (60% reduction at 25 h after the addition of 10 μg/ml). Hence, for the bacteria tested, the inhibition of biofilm formation was not species, strain, or medium specific, and there were no signs of toxicity.

Ursolic acid inhibited *E. coli* biofilm formation in a continuous flow chamber. To further investigate the effect of ursolic acid on biofilm architecture, as well as to corroborate the 96-well plate crystal violet results, a continuous flow chamber was used to establish the biofilm of *E. coli* K-12 ATCC 25404 [R1*drd19*, pCM18]. Since *E. coli* biofilm formation with LB is too robust (data not shown) and it is difficult to see distinct biofilm structure, minimal medium was used to monitor the effect of ursolic acid. The green fluorescent protein plasmid pCM18 was found not to affect the biofilm formation of *E. coli* K-12 ATCC 25404 carrying R1*drd19* for up to 31 h in LB or M9 medium in a 96-well plate biofilm assay (e.g., at 31 h, the OD reading at 540 nm for total biofilm after crystal violet staining was 1.72 for *E. coli* K-12 ATCC 25404 [R1*drd19*, pCM18] and 1.87 for *E. coli* K-12 ATCC 25404 [R1*drd19*]).

Over 65 h, the biofilm development and architecture were monitored in the flow chamber. In the absence of ursolic acid, cells attached to the glass surface and began to make cell clusters within 15 h (data not shown). These cell clusters matured to larger organized microcolonies within 22 h and formed a biofilm 50 μm thick (Fig. 3A). In contrast, when 10 μg of ursolic acid/ml was added, very few microcolonies were seen within 22 h; instead, only single cells or some small dispersed cell clusters were seen (Fig. 3C). Within 65 h, no mature biofilm structure was observed in the presence of ursolic

TABLE 2. Inhibition of *E. coli* K-12 ATCC 25404 [R1*drd19*, pCM18] biofilms by the addition of ursolic acid in flow chambers

Time after inoculation (h)	Conditions	Mean ± SD[a]			
		Biomass ($\mu m^3/\mu m^2$)	Substratum coverage (%)	Mean thickness (μm)	Roughness coefficient
22	No ursolic acid	38 ± 12	40 ± 10	53 ± 20	0.5 ± 0.2
	10 μg of ursolic acid/ml	3 ± 6 (1/10)	2 ± 2 (1/20)	4 ± 9 (1/14)	1.8 ± 0.5 (3.6)
65	No ursolic acid	14 ± 6	10 ± 10	22 ± 7	1.1 ± 0.3
	10 μg of ursolic acid/ml	3.2 ± 2 (1/4)	4 ± 2 (1/2.5)	7 ± 4 (1/3.2)	1.5 ± 0.2 (1.3)

[a] The (10 μg of ursolic acid)/(no ursolic acid) ratios are given in parentheses.

acid, and the biofilm thickness was much less than that without ursolic acid (Fig. 3B and D). COMSTAT analysis of biofilm structure at 22 and 65 h after inoculation confirmed that biofilm development was inhibited by the presence of ursolic acid (Table 2) with significant decreases in biomass, surface coverage, and mean thickness, as well as an increase in the roughness coefficient.

Ursolic acid induces genes for chemotaxis, heat shock, and unknown functions. To investigate the inhibition of biofilm by ursolic acid on a global genetic basis and to compare the findings with our earlier study of another natural biofilm inhibitor furanone (49), DNA microarrays were used here to study the gene expression profiles of *E. coli* K-12 ATCC 25404 grown with or without ursolic acid. These experiments were done in the absence of the conjugative plasmid R1*drd19* so that the results are comparable to the earlier furanone studies and so that the artifact of the roughly 100 genes of R1*drd19* are not introduced. Also, ursolic acid was used with suspension cells since it was not practical to get a reasonable biofilm sample due to the inhibition of biofilm by ursolic acid.

The effects of both 10 and 30 μg of ursolic acid/ml were studied. Each experiment was conducted in duplicate (therefore, there were four sets of DNA microarrays for each concentration of ursolic acid), and a number of genes were found to be induced or repressed consistently (Tables 3 and 4). For the genes affected by ursolic acid, the DNA microarray data of *E. coli* K-12 ATCC 25404 differential gene expression due to furanone and due to AI-2 quorum sensing (49) are also listed in Tables 3 and 4 for comparison. There were 32 genes and 61 genes induced by 10 and 30 μg of ursolic acid/ml, respectively, and 19 genes were consistently induced by both 10 and 30 μg of ursolic acid/ml (Table 3). There was even better consistency in that 11 of the other 13 genes induced by 10 μg of ursolic acid/ml were also upregulated by 30 μg of ursolic acid/ml; they are not listed as induced due to either their high *P* values or because the induction ratios are lower than 1.4 (Table 3). Hence, the upregulation of these genes was consistent, and there were few differences related to the concentration of ursolic acid used with the microarrays.

The induced genes with known functions could be divided into five groups. The first has functions for chemotaxis and motility, such as *cheA*, *motAB*, *tap*, and *tsr*. Although only *cheA* in the *che* operon was consistently induced by 10 and 30 μg of ursolic acid/ml, the other genes in the *che* operon, *cheBWYZ*, were also induced by 10 μg of ursolic acid/ml (Table 3) and upregulated by 30 μg of ursolic acid/ml (all ~1.4-fold, but the *P* values were higher than 0.05; Table 3). The induction of the chemotaxis and motility genes indicated that ursolic acid promotes cell movement. However, ursolic acid appeared to be neither a chemoattractant nor a chemorepellent for *E. coli* based on our chemotaxis assay (data not shown).

The second group of induced genes are involved in a heat shock and stress response such as *hslSTV*, *htpG*, and *mopB*. *hslT* (alternate name *ibpA*) and *hslS* (alternate name *ibpB*) encode small proteins for responding to heat shock and superoxide stresses (30). Hence, the induction of these heat shock genes suggest the cells were under some kind of stress. The third group of induced genes are involved in transport, such as *dcuA*, *emrK*, and *malE* (Table 3). There were also a number of genes for synthesis and metabolism, as well as phage-related genes that were induced by ursolic acid. Nineteen genes with unknown functions were also induced with 10 and/or 30 μg of ursolic acid/ml (Table 3), such as *b1566*, *b1760*, *b2674*, *yjjS*, *yrfH*, and *yrfI*. *b1566* is also known as *ftxA*, and its expression is dependent on the sigma factor for the class 3 flagellar operons; however, the *ftxA* mutant is not deficient in motility (27). *b1566* was also found induced by AI-2 and repressed by furanone in our recent study (49). In addition, we also found recently using DNA microarrays that *b1566* is induced 8.3-fold during *E. coli* K-12 biofilm formation on mild steel plates compared to suspension cells (47). Therefore, this gene may be involved in cell movement and biofilm formation.

Genes repressed by ursolic acid. Based on the microarray results, it was found that 10 and 30 μg of ursolic acid/ml repressed 31 and 17 genes, respectively, and 12 genes were consistently repressed by both 10 and 30 μg of ursolic acid/ml, including *cysDJK* (Table 4) belonging to the cysteine regulon and regulated by CysB (25, 37). Interestingly, *narH* for anaerobic respiration was repressed (Table 4). Since biofilm cells are encountering oxygen limitations (44), the repression of *narH* may help explain biofilm removal by ursolic acid.

Ursolic acid has no effect on the AI-1 and AI-2 quorum-sensing systems. In a recent study, we found that genes for flagellar synthesis, chemotaxis, and motility were induced by AI-2 and repressed by furanone (49) (the expression ratios affected by AI-2 and furanone for the genes induced/repressed by ursolic acid are listed in Tables 3 and 4). Therefore, the induction of chemotaxis genes in the present study suggests a possible interaction between ursolic acid and AI-2 quorum sensing. To test whether ursolic acid stimulates AI-2 quorum sensing via production of AI-2, ursolic acid was added at different concentrations (0, 1, 5, and 10 μg/ml) to cultures of *V. harveyi* BB170 (which senses AI-2), and AI-2 activity was measured. The experiment was conducted in duplicate. No apparent induction of AI-2 activity was observed (relative AI-2 activities of $0.48 \pm 0.4 \times 10^{-5}$ RLU without ursolic acid, 1.0 ±

TABLE 3. *E. coli* K-12 genes induced by ursolic acid[a]

Function and gene	b no.	Ursolic acid 10 μg/ml ER	Ursolic acid 10 μg/ml *P*	Ursolic acid 30 μg/ml ER	Ursolic acid 30 μg/ml *P*	Furanone ER	Furanone *P*	AI-2 ER	AI-2 *P*	Description
Chemotaxis and motility										
air	b3072	1.5	0.0236	1.2	0.2182	−4.6	0.0006	5.3	0.0001	Aerotaxis sensor receptor, flavoprotein
cheA	**b1888**	1.6	0.0017	1.5	0.0112	−22.9	0.0012	13.7	0.0007	Sensory transducer kinase between chemosignal receptors and CheB and CheY
cheB	b1883	1.5	0.0269	1.3	0.2061	−7.7	0.0025	5.7	0.0020	Response regulator for chemotaxis (CheA sensor), protein methylesterase
cheW	b1887	1.6	0.0021	1.5	0.0692	−13.6	0.0037	4.7	0.0020	Positive regulator of CheA protein activity
cheY	b1882	1.5	0.0008	1.4	0.0911	−4.5	0.0018	3.2	0.0000	Chemotaxis regulator transmits chemoreceptor signals to flagellar motor components
cheZ	b1881	1.5	0.0033	1.4	0.0996	−6.5	0.0033	9.0	0.0075	Chemotactic response, CheY protein phosphatase, antagonist of CheY as switch regulator
malE	**b4034**	1.5	0.0035	1.5	0.0462	−2.3	0.0189	1.4	0.0134	Periplasmic maltose-binding protein, substrate recognition for transport and chemotaxis
motA	**b1890**	1.5	0.0028	1.3	0.0024	−10.4	0.0020	5.1	0.0006	Phenotype, chemotaxis, and mobility
motB	**b1889**	1.4	0.0002	1.4	0.0202	−7.7	0.0005	5.1	0.0014	Structural component, chemotaxis, and mobility
tap	**b1885**	1.7	0.0008	1.5	0.0315	−310.2	0.0015	10.2	0.0025	Methyl-accepting chemotaxis protein IV, peptide sensor receptor
tar	**b1886**	1.6	0.0000	1.5	0.0188	−13.3	0.0009	22.5	0.0002	Methyl-accepting chemotaxis protein II, aspartate sensor receptor
Heat shock and stress response										
b0531	b0531	1.7	0.1494	2.3	0.0004	1.2	0.1110	−1.4	0.6103	Putative chaperone
clpB	b2592	1.6	0.0081	2.6	0.0612	1.1	0.0211	−1.8	0.0080	Heat shock protein
dnaJ	b0015	1.5	0.0385	1.8	0.1631	1.0	0.5892	1.1	0.0583	Factor, chaperones
dnaK	b0014	1.6	0.0224	2.1	0.1222	1.1	0.1370	−1.1	0.3628	Chaperone Hsp70, DNA biosynthesis, autoregulated heat shock proteins
grpE	b2614	1.4	0.0474	1.8	0.0412	1.0	0.9806	1.0	0.5185	Phage lambda replication, host DNA synthesis, heat shock protein, protein repair
hslS	**b3686**	2.5	0.0187	6.1	0.0027	2.3	0.2327	−8.5	0.0000	Heat shock protein
hslT	**b3687**	1.6	0.0462	3.0	0.0020	1.4	0.2123	−2.4	0.0000	Heat shock protein
hslU	b3931	1.2	0.0041	2.1	0.0005	1.2	0.1135	−1.4	0.0325	Heat shock protein Hs1VU, ATPase subunit, homologous to chaperones
hslV	**b3932**	1.7	0.0285	2.0	0.0085	1.1	0.7736	−1.3	0.2388	Heat shock protein HslVU, proteasome-related peptidase subunit
htpG	**b0473**	1.9	0.0042	2.5	0.0093	1.2	0.1991	−1.1	0.3880	Chaperone Hsp90, heat shock protein C 62.5
lon	b0439	1.4	0.0016	1.5	0.0154	1.0	0.2636	−1.1	0.5716	Heat shock K protein
mopA	**b4143**	1.4	0.0310	2.3	0.0004	1.2	0.0152	1.0	0.3708	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein
mopB	**b4142**	1.5	0.0192	2.4	0.0039	1.2	0.0170	−1.1	0.6626	GroES, 10-kDa chaperone
yrfH	**b3400**	1.7	0.0002	2.5	0.0019	1.2	0.0039	−1.9	0.0034	Hsp15, DNA/RNA binding heat shock protein
yrfI	**b3401**	1.5	0.0011	1.9	0.0011	1.2	0.1008	−1.8	0.0023	Hsp33, redox regulated chaperone
emrK	**b2368**	1.5	0.0094	1.9	0.0097	1.3	0.0037	−1.1	0.6719	Multi-drug resistance protein K
arsC	b3503	−1.6	0.0446	1.4	0.0143	2.3	0.2574	−1.3	0.8110	Enzyme, drug/analog sensitivity
cutA	b4137	1.1	0.9459	1.5	0.0127	1.4	0.1734	−1.1	0.2734	Divalent cation tolerance protein, cytochrome *c* biogenesis
soxS	b4062	1.2	0.3597	1.4	0.0069	2.1	0.3581	−3.1	0.0031	Regulation of superoxide response regulon
Transport										
cycA	b4208	1.5	0.0005	1.4	0.0170	1.0	0.3535	−1.4	0.0073	Transport of D-alanine, D-serine, and glycine
dcuA	**b4138**	1.6	0.0074	1.5	0.0004	1.0	0.3122	1.0	0.4645	Transport of small molecules: carbohydrates, organic acids, alcohols
b1312	b1312	1.0	1.0000	1.6	0.0319	1.5	0.6014	−2.0	0.3356	Putative transport system permease protein
bglF	b3722	1.4	0.0546	1.6	0.0053	1.0	0.5143	−1.6	0.1040	PTS system β-glucosides, enzyme II, cryptic
fhuD	b0152	1.1	0.8560	1.6	0.0032	1.4	0.9189	−2.1	0.4068	Hydroxamate-dependent iron uptake, cytoplasmic membrane component
nikE	b3480	1.0	0.5591	1.5	0.0012	1.0	0.3503	1.0	0.2808	ATP-binding protein of nickel transport system
yhaO	b3110	1.4	0.1321	2.4	0.0018	−2.0	0.0184	−1.7	0.1292	Putative transport system permease protein
yhjX	b3547	1.1	0.6883	1.4	0.0003	1.1	0.0693	−1.5	0.1064	Putative transport, drug/analog sensitivity
Synthesis and metabolism										
b2673	**b2673**	1.4	0.0042	2.0	0.0023	3.6	0.2100	−1.8	0.7355	Enzyme, biosynthesis of cofactors, carriers: thioredoxin, glutaredoxin, glutathione

Continued on facing page

TABLE 3—*Continued*

Function and gene	b no.	Ursolic acid				Furanone		AI-2		Description
		10 μg/ml		30 μg/ml						
		ER	*P*	ER	*P*	ER	*P*	ER	*P*	
fdoI	b3892	1.6	0.0006	−1.1	0.4060	1.1	0.4964	−1.3	0.1909	Energy metabolism, carbon: anaerobic respiration
entC	b0593	1.5	0.1515	1.5	0.0055	1.8	0.2511	−6.9	0.0936	Isochorismate hydroxymutase 2, enterochelin biosynthesis
hycI	b2717	1.3	0.0434	1.7	0.0103	1.2	0.1496	−1.4	0.0875	Enzyme, degradation of proteins, peptides, glyco
ldhA	b1380	1.0	0.7186	1.7	0.0008	1.1	0.4002	−1.1	0.6766	Energy metabolism, carbon: fermentation
narZ	b1468	1.0	0.8904	1.4	0.0057	1.8	0.1196	1.2	0.1316	Energy metabolism, carbon: anaerobic respiration
miaA	b4171	1.4	0.0002	1.7	0.0189	1.1	0.4461	−1.1	0.6388	Aminoacyl tRNA synthetases, tRNA modification
nrdE	b2675	1.4	0.0807	1.5	0.0143	1.5	0.0029	−2.0	0.0251	Ribonucleoside-diphosphate reductase 2, alpha subunit
pdhR	b0113	1.3	0.0114	2.0	0.0373	1.3	0.1203	1.3	0.0125	Transcriptional regulator for pyruvate dehydrogenase complex
yhgJ	b3419	1.2	0.0509	1.4	0.0256	1.0	0.5413	−1.5	0.0019	Enzyme, RNA synthesis, modification, DNA transcription
yhgK	b3420	1.3	0.0847	1.6	0.0016	1.1	0.2198	−1.7	0.0035	Enzyme, RNA synthesis, modification, DNA transcription
Phage-related genes										
b0281	b0281	1.2	0.0279	1.4	0.0287	1.2	0.0206	−1.1	0.7089	Putative phage integrase
pspA	b1304	1.0	0.6835	1.5	0.0057	1.7	0.0033	−1.3	0.5019	Phage shock protein, inner membrane protein
pspB	b1305	1.2	0.0197	1.6	0.0021	1.4	0.2287	−1.9	0.6609	Phage shock protein
pspD	b1307	1.0	0.9586	1.4	0.0035	2.0	0.2919	−2.6	0.8178	Phage shock protein
yi81_2	b0582	1.3	0.0010	1.4	0.0026	1.0	0.7197	−1.1	0.6902	IS, phage, Tn, transposon-related functions
yi82_1	b0017	1.3	0.0257	1.5	0.0012	1.0	0.9523	−1.7	0.2159	IS, phage, Tn, transposon-related functions
Genes with other functions and unknown functions										
cirA	b2155	1.4	0.2005	1.6	0.0011	1.3	0.9818	−5.7	0.0001	Membrane, outer membrane constituents
entE	b0594	1.1	0.3910	1.8	0.0198	1.5	0.2639	−2.6	0.0241	2,3-Dihydroxybenzoate-AMP ligase
fxsA	b4140	1.4	0.0043	1.7	0.0017	1.0	0.4058	−2.9	0.9522	Suppressor of F exclusion of bacteriophage T7
mlc	b1594	1.3	0.0142	1.4	0.0157	1.2	0.6067	−1.2	0.8621	Putative NAGC-like transcriptional regulator
osmE	b1739	1.8	0.0004	1.0	0.8746	1.1	0.2632	1.0	0.4443	Transcriptional activator of *ntrL* gene
prlC	b3498	1.4	0.0951	1.7	0.0016	1.3	0.0134	−2.0	0.0024	Oligopeptidase A
b1194	b1194	1.6	0.0100	1.8	0.0778	−4.0	0.0105	6.3	0.0003	Orf, hypothetical protein
b1321	b1321	1.3	0.0023	1.5	0.0023	1.0	0.4546	1.0	0.8155	Putative enzyme, not classified
b1566	**b1566**	1.5	0.0105	1.6	0.0431	−7.1	0.0061	8.8	0.0002	ORF, hypothetical protein
b1760	**b1760**	1.6	0.0075	1.7	0.0108	−2.4	0.0398	4.9	0.0091	ORF, hypothetical protein
yadN	b0141	1.4	0.0351	1.6	0.0003	1.1	0.5917	−1.2	0.1473	Putative fimbrial-like protein
b2674	**b2674**	1.5	0.0303	1.8	0.0032	1.9	0.0345	−2.6	0.2658	ORF, hypothetical protein
ybeD	b0631	1.0	0.7382	1.5	0.0080	1.1	0.4518	−1.1	0.0123	ORF, hypothetical protein
ycjF	b1322	1.2	0.0881	1.6	0.0010	1.3	0.0114	1.1	0.2333	ORF, hypothetical protein
yeiE	b2157	1.3	0.0127	1.5	0.0023	1.0	0.4394	−1.4	0.4110	Putative regulator, not classified
yhdM	b3292	1.0	0.6785	1.4	0.0460	1.4	0.8804	−2.0	0.0308	Zn(II)-responsive transcriptional regulator
yhjH	b3525	1.6	0.1570	1.5	0.0169	−9.9	0.0000	6.0	0.0002	ORF, hypothetical protein
yjjS	**b4367**	1.5	0.0266	1.6	0.0145	1.1	0.0891	−1.2	0.1999	ORF, hypothetical protein
yrfG	b3399	1.4	0.0601	1.7	0.0120	−1.1	0.2017	−1.8	0.0179	Putative enzyme, not classified

[a] For comparison, differential expression is indicated for the effect of AI-2 and 60 μg/ml furanone (49). The underlined ratios indicate the corresponding genes were significantly induced by ursolic acid. The genes in boldface were induced by both 10 and 30 μg/ml ursolic acid. ER, expression ratio.

0.2×10^{-5} RLU with 5 μg of ursolic acid/ml, and $0.6 \pm 0.005 \times 10^{-5}$ RLU with 10 μg of ursolic acid/ml). In contrast, the positive control showed an induction of 3,400-fold.

To understand whether ursolic acid inhibits AI-2 quorum sensing, cell-free supernatants from *V. harveyi* BB120 containing AI-2 were added to *V. harveyi* BB170 cultures as 10% (vol/vol), and the effect of ursolic acid at 0, 1, 5, 10, and 30 μg/ml was studied (an individual control sample was used for each ursolic acid concentration which had the same amount of ethanol as the corresponding ursolic acid sample). No apparent effect on AI-2 activity was found (e.g., relative AI-2 activity was 0.0034 RLU without ursolic acid and 0.0040 RLU with 10 μg of ursolic acid/ml). In comparison, we showed previously that *E. coli* AI-2 was repressed by 26,600-fold by furanone (51). Therefore, ursolic acid neither induces nor represses AI-2 quorum sensing.

Similarly, the effect of ursolic acid on *V. harveyi* AI-1 quorum sensing was also studied by using *V. harveyi* BB886 as a re-

TABLE 4. *E. coli* K-12 genes repressed by ursolic acid[a]

Gene	b no.	Ursolic acid				Furanone		AI-2		Description
		10 μg/ml		30 μg/ml						
		ER	*P*	ER	*P*	ER	*P*	ER	*P*	
Genes with known functions										
arsC	b3503	−1.5	0.0446	1.4	0.0143	2.3	0.2574	−1.3	0.8110	Enzyme, drug/analog sensitivity
b2789	b2789	−1.9	0.0557	−2.5	0.0377	11.8	0.7871	−66.9	0.1484	Putative D-glucarate permease (MFS family)
cspF	b1558	−1.6	0.0033	−1.1	0.3568	1.8	0.3239	−8.6	0.2342	Cold shock-like protein
cspG	**b0990**	−2.5	0.0085	−1.7	0.0166	2.5	0.0011	1.3	0.2950	Homolog of *Salmonella* cold shock protein
cysB	b1275	−1.7	0.0377	−1.4	0.0175	1.1	0.6877	1.4	0.0013	Positive transcriptional regulator for cysteine regulon
cysD	**b2752**	−2.5	0.0009	−2.5	0.0240	1.2	0.2733	1.7	0.1271	Central intermediary metabolism: sulfur metabolism
cysI	b2763	−1.5	0.0692	−1.7	0.0002	1.0	0.7077	2.5	0.0002	Central intermediary metabolism: sulfur metabolism
cysJ	**b2764**	−3.6	0.0147	−3.3	0.0090	1.2	0.3507	2.5	0.0024	Central intermediary metabolism: sulfur metabolism
cysK	**b2414**	−3.6	0.0026	−3.3	0.0079	1.0	0.2691	2.5	0.0002	Amino acid biosynthesis: cysteine
frvR	b3897	−5.4	0.0061	−2.0	0.1754	1.7	0.5280	3.3	0.0004	Putative *frv* operon regulatory protein
gntU_1	b3436	−1.5	0.0257	−1.4	0.0428	4.3	0.8882	2.0	0.1005	Transport of small molecules: carbohydrates, organic acids, alcohols
narH	**b1225**	−1.6	0.0022	−1.4	0.0281	1.5	0.0521	2.0	0.0029	Energy metabolism, carbon: anaerobic respiration
pheM	b1715	−1.6	0.0106	1.0	0.7617	1.3	0.5432	1.0	0.9909	Aminoacyl tRNA synthetases, tRNA modification
pheP	b0576	−1.5	0.0210	−1.1	0.4985	1.2	0.6364	1.3	0.4968	Transport of small molecules: amino acids, amines
rimL	b1427	−1.5	0.0220	1.0	0.7185	1.3	0.5425	−1.6	0.2914	Enzyme, ribosomes: maturation and modification
rmf	b0953	−1.5	0.0029	1.0	0.6617	1.4	0.8536	−1.8	0.0964	Factor, ribosomes: maturation and modification
rpmI	b1717	−1.6	0.0072	1.0	0.7076	−1.1	0.3271	−1.1	0.7165	Structural component, ribosomal proteins: synthesis, modification
slp	**b3506**	−1.5	0.0059	−1.6	0.0020	2.5	0.1058	−6.5	0.0013	Outer membrane constituents
ugpB	b3453	−1.4	0.0446	−1.5	0.0210	2.2	0.0098	−2.7	0.0026	Transport of small molecules: carbohydrates, organic acids, alcohols
ybiK	**b0828**	−2.4	0.0007	−2.2	0.0051	1.2	0.2979	1.7	0.0103	Putative asparaginase
yhaD	**b3124**	−1.6	0.0254	−2.6	0.0091	2.2	0.8505	−2.3	0.4040	Glycerate kinase I
yhaF	**b3126**	−1.5	0.0089	−2.4	0.0023	1.2	0.2202	−1.6	0.0043	alpha-dehydro-beta-deoxy-D-glucarate aldolase
yhaG	**b3128**	−2.0	0.0039	−2.2	0.0077	1.1	0.4891	−2.1	0.1735	(D)-Galactarate dehydrogenase
Genes with unknown functions										
b0309	b0309	−1.7	0.0420	−1.3	0.1550	2.6	0.7326	16.7	0.4347	ORF, hypothetical protein
b0484	b0484	−1.5	0.0435	−1.1	0.4246	1.7	0.0298	−1.4	0.1332	Putative enzyme, not classified
b0485	b0485	−1.8	0.0092	−1.3	0.0186	1.8	0.1369	−1.6	0.0503	Putative enzyme, not classified
b0829	b0829	−1.5	0.0315	−1.5	0.0898	1.0	0.5829	1.3	0.0213	Putative transport, not classified
b1729	b1729	−5.6	0.0029	−2.0	0.1330	1.5	0.5753	3.3	0.0020	Putative enzyme, not classified
b2379	b2379	−1.5	0.0114	1.0	0.3246	1.6	0.0072	1.0	0.5700	Putative enzyme, not classified
hdeA	**b3510**	−1.7	0.0075	−1.4	0.0077	2.9	0.0010	−13.2	0.0000	ORF, hypothetical protein
hdeB	**b3509**	−1.8	0.0006	−1.4	0.0102	3.9	0.0002	−17.8	0.0000	ORF, hypothetical protein
yeeD	b2012	−2.3	0.0253	−1.4	0.2278	1.3	0.6484	2.5	0.0277	ORF, hypothetical protein
yeeE	b2013	−12.6	0.0058	−2.0	0.1816	1.1	0.9239	3.3	0.0079	Putative transport, not classified
yjeB	b4178	−1.4	0.0051	1.0	0.8329	1.5	0.0369	−1.7	0.0466	ORF, hypothetical protein
ybhG	b0795	−1.4	0.0134	−1.4	0.0020	2.8	0.2822	−1.4	0.5428	Putative membrane, not classified
yhaU	b3127	−1.9	0.0744	−4.2	0.0030	18.9	0.8100	−1.2	0.3398	Putative transport protein

[a] For comparison, differential expression is indicated for the effect of AI-2 and 60 μg/ml furanone (49). The underlined ratios indicate the corresponding genes were significantly repressed by ursolic acid. The highlighted genes were repressed both by 10 and 30 μg/ml ursolic acid. ER, expression ratio.

porter. This experiment was conducted in duplicate. Again, ursolic acid did not induce (relative AI-1 activities of $4.5 \pm 3.0 \times 10^{-4}$ RLU without ursolic acid and $4.2 \pm 3.2 \times 10^{-4}$ RLU with 10 μg of ursolic acid/ml) or repress (relative AI-1 activities $6.8 \pm 0.1 \times 10^{-5}$ without ursolic acid and $7.8 \pm 2.0 \times 10^{-3}$ with 10 μg of ursolic acid/ml) the AI-1 activity. In contrast, the positive control showed an induction of 17-fold. Hence, the induction of chemotaxis and motility genes by ursolic acid was

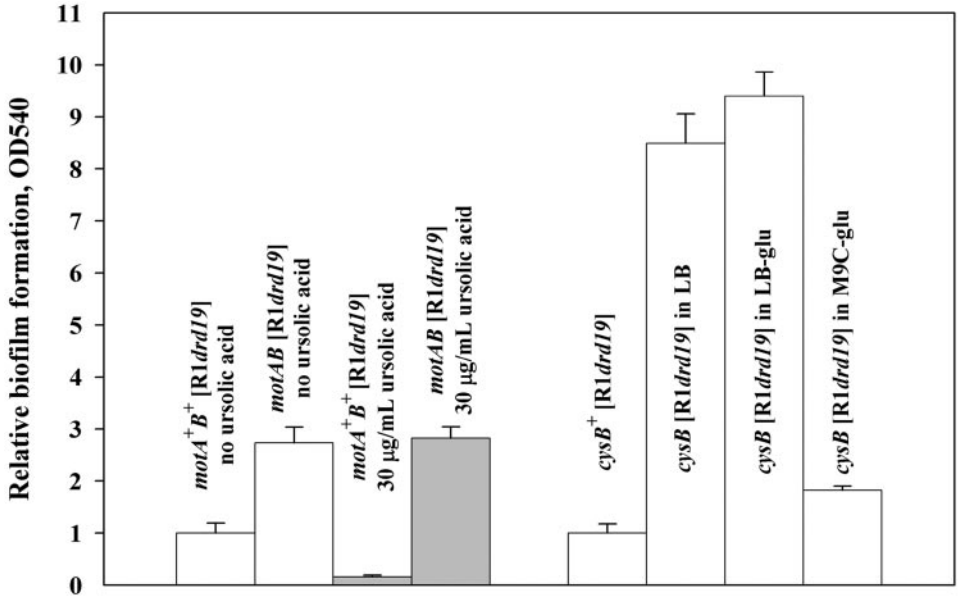

FIG. 4. Effect of the *E. coli motAB* mutation on ursolic acid biofilm inhibition (*E. coli* K-12 MG1655 [R1*drd19*] versus *E. coli* K-12 MG1655 *motAB* [R1*drd19*], data collected 24 h after addition of 30 μg of ursolic acid/ml in LB medium), and the effect of the *E. coli cysB* mutation on biofilm formation (*E. coli* K-12 MG1655 [R1*drd19*] versus *E. coli* K-12 MG1655 *cysB* [R1*drd19*], data collected 24 h after inoculation in LB, LB supplemented with 0.2% glucose, and M9 minimal medium supplemented with 0.4% glucose and 0.4% Casamino Acids). Biofilm mass was normalized based on the reading of wild type without ursolic acid. One standard deviation shown.

not through the interaction with either the AI-1 or the AI-2 quorum-sensing system. Note the ursolic acid did not inhibit the growth of the *Vibrio* strains in LB medium.

***motAB* and *cysB* affect biofilm formation.** Since the DNA microarrays identified that *motAB* was induced by ursolic acid (Table 3), we tested strains with mutations in these genes in the presence of ursolic acid to corroborate these results. Note that the *motAB* mutation causes cells to lack flagellar motion but not synthesis (5, 43). Without the conjugation plasmid R1*drd19*, deletion of *motAB* caused the cells to decrease biofilm formation 1.6-fold relative to the isogenic $motA^+B^+$ strain as originally reported (43); however, in the presence of the conjugation plasmid R1*drd19*, the strain with the *motAB* deletion made 2.7-fold more biofilm than its parent strain (Fig. 4), suggesting that low motility is favorable for conjugation which promotes biofilm development (16, 45). The results also showed that, compared to its isogenic $motA^+B^+$ strain, the *motAB* mutant is much less sensitive to ursolic acid (in terms of biofilm inhibition), i.e., in the presence of R1*drd19* there was no biofilm inhibition with 30 μg of ursolic acid/ml for the *motAB* mutant versus 84% biofilm reduction for $motA^+B^+$ strain (Fig. 4). This is expected according to the DNA microarray data because ursolic acid decreases biofilm formation by inducing expression of *motAB*, making cells too motile to stay in the biofilm community stably, whereas removing *motAB* simply counteracts the ability of ursolic acid to inhibit biofilms since the cells are paralyzed. There was no effect of the *motAB* mutation on the growth of *E. coli* K-12 MG1655 in LB medium.

Since the microarray data indicated that the *cysB*-regulated operon *cysDJK* was repressed by ursolic acid (Table 4), we also studied biofilm formation by a *cysB* mutant. Interestingly, we found that the *cysB* mutation increased biofilm formation by 2- to 10-fold compared to its isogenic $cysB^+$ strain in all three media tested (Fig. 4). The observation that the *cysB* mutation affects biofilm formation was corroborated by two other *cysB* mutants versus their respective $cysB^+$ hosts (*E. coli* MC4100 *cysB* [R1*drd19*] in both LB medium and LB plus 0.2% glucose and *E. coli* C600 *cysB* [R1*drd19*] in LB medium). Although it is not explained by the microarray data, the result that the *cysB* mutation increases biofilm formation suggests that CysB controls biofilm formation in a way different from ursolic acid. We noted that *cysB* mutation decreased specific growth rates of *E. coli* K-12 MG1655 by 30% in LB and LB containing 0.2% glucose but not in M9 medium supplemented with 0.4% glucose and 0.4% Casamino Acids. This defect in growth by *cysB* mutation is not a concern for biofilm formation since biofilm is actually increased by the *cysB* mutant (in the absence of ursolic acid).

DISCUSSION

We focused on *E. coli* biofilms since this strain is the most thoroughly studied bacterium (6), its genome is sequenced (6), microarrays are available (47, 56), the function of many of its proteins have been elucidated (52), and many isogenic mutants are available (17). Also, our group has experience in determining the genetic basis of *E. coli* biofilm formation and its inhibition with a natural plant-derived antagonist (47, 51). Furthermore, although *E. coli* is well studied, its biofilm has not received the same scrutiny since strain K-12 makes a poor biofilm if it lacks a conjugative plasmid (16, 45). Hence, the results of our study are helpful for understanding and preventing the biofilm of the archetypal strain, as well as helpful for combating pathogenic strains such as *E. coli* O157:H7 (41).

In the present study, we identify a new chemical class of

biofilm inhibitor, ursolic acid, and show it is completely nontoxic to *E. coli*, *P. aeruginosa* PAO1, *V. harveyi*, and hepatocytes. Moreover, biofilm inhibition was seen in five different *E. coli* hosts (K-12, JM109, C600, EJ500, and JCB495) and in four different media (M9, LB, and LB supplemented with either 0.2% glucose or 2% sodium pyruvate); hence, the biofilm inhibition by ursolic acid does not seem to be restricted to specific species, strains, or growth conditions.

By studying the complete transcriptome, it was also shown that ursolic acid induces chemotaxis, motility, and heat shock genes in *E. coli* and represses genes related to sulfur metabolism. Also, ursolic acid inhibits the development of mature biofilms both in LB medium grown in the 96-well plate assay and in M9 medium (0.4% glucose and 0.4% Casamino Acids) in a continuous flow chamber. The exact mechanism of inhibition is not clear, but the induction of chemotaxis and motility genes suggests that ursolic acid may function as a signal that tells cells to remain too motile for adequate biofilm formation. It has been reported that motility is important for initial cell attachment in *E. coli* biofilm formation (43). For biofilm maturation, however, the cells need to stay attached to the surface and produce the polysaccharide matrix (67); hence, the motility genes may have to be coordinately regulated since regulation has been shown to be important for other biofilm-related genes (28, 61). Therefore, the induction of chemotaxis and motility genes during the wrong stage of biofilm development may destabilize the biofilm and prevent the formation of a mature biofilm. However, ursolic acid itself does not appear to be a chemoattractant or a chemorepellant for *E. coli*, and a direct relationship between ursolic acid and motility was not found in that both a motility assay with 30 μg of ursolic acid/ml present in soft agar plates (59) and β-galactosidase reporter assays with 30 μg of ursolic acid/ml to check induction of the motility-related promoters *qseB*, *fliC*, *motA*, *flhD*, and *fliAeh* (59) did not show an impact by ursolic acid. Since *mopA* and *mopB* are induced by ursolic acid (Table 3) and since MopA and MopB are chaperons used to refold damaged proteins (38), ursolic acid may disrupt a negative regulator of motility.

Here, biofilm formation was not affected by ursolic acid with a strain that is paralyzed with the *motAB* mutation, which indicates the importance of torque generation for biofilm inhibition by ursolic acid. These results (Fig. 4) also indicate again that motility is not necessary for normal biofilm formation in *E. coli* in the presence of a conjugation plasmid (45), although it has been found to alter the biofilm structure in *P. aeruginosa* PAO1 (31). Note that there was less biofilm with the *motAB* mutation than with the wild-type strain (without the conjugation plasmid R1*drd19*) in the absence of ursolic acid as expected (43).

Previously, we have shown that furanone inhibited genes related to AI-2 quorum sensing (49), that it inhibited biofilm formation of *E. coli* (51), and that it negatively regulated chemotaxis genes (49) (see Tables 3 and 4 for the gene expression ratios for genes in common with those affected by ursolic acid). Hence, it appears that ursolic acid and furanone both inhibit biofilm formation, but by controlling the chemotaxis genes in an opposite manner and therefore affecting different stages of biofilm formation.

It is not clear whether the plants produce ursolic acid constitutively or only after infection by a pathogen. Given that ursolic acid induced chemotaxis genes, ursolic acid may be a defense response of the host after the infection of the pathogen. Further study of the surface concentration of ursolic acid on plants and the effect of environmental conditions on its production may be informative.

Although the microarray data in the present study are statistically significant since the cutoff ratio was based on a standard deviation higher than 2.5 and the P values were <0.05 for the induced and repressed genes, the induction and repression ratios of microarray results in the present study (1.5- to 2-fold; see Tables 3 and 4) are modest compared to our earlier microarray experiments. For example, our previous study showed that 5 μg of furanone/ml induced 94 genes of *Bacillus subtilis* >5-fold as it acted as a novel antimicrobial for this gram-positive species (48). Another recent study of ours in which furanone acts as a nontoxic biofilm inhibitor for gram-negative strains (49) showed that 60 μg/ml repressed the chemotaxis and motility genes 7- to 300-fold. Although modest, these ursolic acid microarray results are robust in that they provided important insights, as evidenced by the discovery that deleting *motAB* counteracts biofilm inhibition by ursolic acid and the discovery that sulfur metabolism (through *cysB*) affects biofilm formation.

Like furanone, the ursolic acid was effective at low concentrations since 10 μg/ml removed 72% of the *E. coli* JM109 biofilm. Hence, the addition of ursolic acid to destabilize biofilms is a promising approach. The structure of ursolic acid may also be altered to improve efficacy by using combinatorial chemistry and screening with the biofilm assay to identify even better biofilm inhibitors. It is also clear that by studying the molecular basis of the inhibition of biofilms with plant-derived antagonists, important discoveries may be made about the nature of biofilm formation. Here, it was found for the first time that sulfur metabolism is important for biofilms.

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